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TITLE: SYSTEM AND METHOD FOR INTRODUCTION AND STABILIZATION OF GENES IN *THERMUS* SP.

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SYSTEM AND METHOD FOR INTRODUCTION AND
STABILIZATION OF GENES IN *THERMUS* SP.

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5 Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

Field of the Invention

This invention is related to a method and system for introducing and
stabilizing heterologous and recombinant genes in *Thermus* sp. This invention further

10 relates to host-vector systems for *Thermus* sp. based upon characteristic genes that
define a detectable host characteristic. More particularly, this invention relates to
host-vector systems for *Thermus* sp. based upon the malate dehydrogenase gene, the
phytoene dehydrogenase gene and the beta-galactosidase gene.

Thermus sp. are thermophilic microorganisms that can survive in

15 extreme environments with temperatures as high as 90°C. Because of the high
growth temperature, reaction rates are faster and thermostable enzymes/proteins are
more durable/resilient in industrial processes than mesophilic enzymes/proteins.

High level expression of cloned genes is often achieved in *E. coli*,
Bacillus subtilis and other well studied microorganisms by the use of high copy

20 number plasmid vectors. Besides high copy number, plasmid expression vectors have
an advantage in comparison with integrative vectors of being easier to recover/purify
from the host strain so that subsequent genetic modifications can be conveniently
performed. However, plasmids are less stable than genes integrated into the

chromosome of a host and plasmid instability is thought to be of particular concern in *Thermus* hosts where growth temperatures of 55°C to 82°C are routine. Another disadvantage of plasmid versus integrative vectors for use in *Thermus* sp. is that plasmid vectors generally have from 10 to 1,000-fold lower transformation efficiencies than integrative vectors. Consequently, integrative vectors are frequently used in genetic studies of *Thermus* sp.

Plasmid vectors that have been used in *Thermus* sp. have been constructed that encode tryptophan, leucine or pyrimidine synthesis genes to complement auxotrophic and deleted hosts, thereby providing for positive selection of transformants. However, such expression vectors were cumbersome to use because they do not provide a wide choice of convenient cloning sites, choice of promoters and ribosomal binding sites, nor do they provide a convenient in vivo means of monitoring transcription of the cloned gene(s).

SUMMARY OF THE INVENTION

Accordingly, it is one object of this invention to provide a method and system for convenient in vivo means of monitoring transcription of cloned genes in *Thermus* sp. By "convenient", we mean methods and systems employing vectors which provide a wide choice of convenient cloning sites, a choice of promoters and ribosomal binding sites, visual confirmation of transformation, and simple quantification of gene expression.

This and other objects of this invention are addressed by a method for introducing and stabilizing heterologous and recombinant genes in a thermophilic host

in which a characteristic gene defining a detectable host characteristic is inactivated in or deleted from the thermophilic host, resulting in a modified thermophilic host expressing an absence of the detectable host characteristic. A DNA fragment of interest is introduced into the modified thermophilic host together with an intact said

5   characteristic gene, whereby the detectable host characteristic is restored to the thermophilic host, thereby enabling detection and confirmation of successful transformation of a plasmid or integration of the DNA fragment into the chromosome of the thermophilic host. The detectable host characteristic is deleted or diminished in activity by suitable integration vectors. Colonies resulting from transformation of

10   these vectors into wild type *Thermus* cultures are recognized by an insertional inactivation of the detectable host characteristic. Such modified cultures can then be used as recipients in future transformation experiments.

In accordance with one embodiment of this invention, an integrative vector is employed that deletes or diminishes the malate dehydrogenase (*mdh*) gene

15   activity in *Thermus* sp. Colonies resulting from transformation of this vector into wild type *Thermus* cultures are recognized by an insertional inactivation of the malate dehydrogenase gene that yields a "sick" colony phenotype. In accordance with another embodiment of this invention, an integrative vector is employed that deletes or diminishes phytoene dehydrogenase gene activity in *Thermus* sp. Colonies

20   resulting from transformation of this vector into wild type *Thermus* cultures contain deleted or inactivated dehydrogenase genes, which can only be white in color and which are recognized by changes in color relative to those colonies in which the

phytoene dehydrogenase gene is intact. In accordance with yet another embodiment

of this invention, vectors that delete or diminish the activity of the *Thermus* beta-

galactosidase gene are employed and colonies resulting from transformation of this

vector are also recognized by differences in color as well as by enzymatic assays.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other objects and features of this invention will be better

understood from the following detailed description taken in conjunction with the

drawings wherein:

Fig. 1 is a diagram showing the method for introducing and stabilizing

10   heterologous and recombinant genes in a thermophilic host in accordance with one

embodiment of this invention. More particularly, Fig. S is a schematic illustration of

stabilizing a plasmid containing an *mdh* gene by use of a *Thermus* host that lacks an

mdh gene;

Fig. 2 is a diagram showing the construction of *Thermus thermophilus*

15   MM8-5 that contains a deletion of the *mdh* gene. In step A, a PCR fragment

containing the *T. flavus mdh* gene along with several hundred base pairs of 5' and 3'

flanking regions (scsA and gpt, respectively) is cloned into pUC18 to create pUC-

SMA. Then, in step B, a DNA fragment containing a kanamycin resistance gene

under the control of a *Thermus* promoter, J17, is cloned into pUC-SMA, replacing the

20   BsiWI/NgoMI fragment that contains the *mdh* gene to create pUC-SKmA, which, in

step C, is introduced into *T. thermophilus* where a double crossover homologous

r5ecombination results in the replacement of the chromosomal *mdh* gene with a

kanamycin resistance gene;

Fig. 3 shows the construction of plasmid and integrative vectors for expression of the *mdh* gene in *Thermus* species. Part A illustrates pTEXI *mdh*, which is a plasmid capable of replicating in *Thermus* and *E. coli* and which contains a complete *mdh* gene under the control of *Thermus* promoter J17 and a *Thermus* ribosome binding site. A transcriptional terminator is located just upstream from promoter J17. Part B illustrates integrative vector *pSJ17mdhA*, which can replicate in *E. coli* but not *Thermus*. It contains the *mdh* gene under the control of *Thermus* promoter J17, and the *mdh* contains several hundred base pairs of 5' and 3' flanking sequences (*scgA* and *gpt*, respectively) that allow homologous recombination with sequences in the *Thermus* chromosome when *pSJ17mdhA* is introduced into a *Thermus* host;

Fig. 4 illustrates the construction of an integrative vector used to delete the *phyD* gene from the chromosome of *Thermus* sp. More particularly, Fig. 4 shows the cloning of a PCR fragment containing the *Thermus phyD* gene into *E. coli* vector *pGEMT-easy* to create *pGEM-phyD*, which was digested with *BglII* and *KpnI* to remove a control portion, but not all, of the *phyD* gene to yield *pGEM-phyD trunc*. When *pGEM-phyD trunc* is introduced into *Thermus*, homologous recombination allows insertional inactivation or deletion of a portion of the *phyD* gene in the chromosome of a *Thermus* host, which can be recognized by colonies that are devoid of pigmentation;

Fig. 5 shows the construction of a plasmid used to express the *phyD*

gene in *Thermus* hosts in accordance with this invention. More particularly, Fig. 5

illustrates *pTEX1-phyD* which contains genes allowing replication in both *E. coli* and

Thermus and contains the *phyD* gene under the control of *Thermus* promoter J17;

Fig. 6 illustrates the construction of *Thermus thermophilus* PPKU that

5   contains a deletion of the β-galactosidase gene. More particularly, Fig. 6 shows the

cloning of a 1968 bp PCR fragment containing the β-galactosidase gene along with

several hundred base pairs of 3' and 5' flanking sequences into *E. coli* plasmid *pUC18*

to create *pUC 18P-G-U*. Plasmid *pUC18P-G-U* is then digested with *EcoRI* and *KpnI*

to remove a 1320 bp fragment containing the β-galactosidase gene, which is replaced

10   by a 1019 bp fragment containing the kanamycin resistance gene under the control of

Thermus promoter J17 to yield *pUC18P-Km-U*. When *pUC18P-Km-U* is introduced

into a *Thermus* host, a double crossover homologous recombination results in the

deletion of the chromosomal β-galactosidase gene and its replacement with the

kanamycin resistance gene;

15   Fig. 7 shows the construction of a plasmid used to express β-

galactosidase in *Thermus* species in accordance with one embodiment of this

invention. More particularly, Fig. 7 shows the construction of a hybrid replicon

resulting from the combination of the *Thermus/E.coli* shuttle vector *pMK18* with *E.*

coli plasmid *pUC18* and PCR fragments containing the *Thermus* β-galactosidase gene

20   and a *Thermus* promoter (*PdnaK, Parg, Pscs-mdh,* or other promoters) to yield

pTEX5β-gal, or a related plasmid. Subsequently, the kanamycin resistance gene

under the control of a *Thermus* promoter is added to the vector to facilitate the

isolation of transformants in *Thermus*;

Fig. 8 illustrates the levels of β-galactosidase produced by *T. thermophilus* HB27 with and without *pTEX9*, a plasmid containing the β-galactosidase gene under the control of the promoter *Pscs-mdh*. The level of β-

5　galactosidase is increased in cells containing *pTEX9* and levels vary in different carbon sources reflecting the inducibility of the *Pscs-mdh* promoter; and

Fig. 9 is a diagram showing the induction of β-galactosidase activity of the *Parg::β-gal* fusion in *pTEX8* by increasing arginine concentration in the growth medium. The plasmid *pTEX8* contains the β-galactosidase gene under the control of

10　the *Parg* promoter, which is induced by arginine up to a concentration of 30mM, but higher arginine concentrations decrease the activity of this promoter.

DETAILED DESCRIPTION OF THE PRESENTLY
PREFERRED EMBODIMENTS

The invention disclosed and claimed herein is a method for introducing

15　and stabilizing heterologous and recombinant genes in thermophilic hosts employing host-vector systems comprising a thermophilic host strain in which a detectable host characteristic gene is inactivated or deleted resulting in a change in the host strain phenotype and an integrative or plasmid vector comprising an intact detectable characteristic gene and a gene of interest for insertion into the thermophilic host.

20　Deletion of the mdh locus is accomplished by constructing a kanamycin resistance gene cassette in which the kanamycin gene is flanked by DNA sequences homologous to those DNA sequences that flank the *mdh* gene in the *Thermus* chromosome. The

length of these flanking homologous regions is from 50 bp to 1,000 bp or more. The kanamycin resistance gene cassette has no ability to replicate autonomously in *Thermus* so when it is introduced into *Thermus* by natural transformation or electroporation selection for kanamycin resistant transformants results in the isolation of cultures in which homologous recombination has resulted in the deletion of the *mdh* gene and substitution of the kanamycin resistance gene as shown in Figure 1.

In accordance with one embodiment of the method of this invention as shown in Fig. 1, the detectable characteristic gene is the malate dehydrogenase gene, which, when rendered inactive in the *Thermus* sp., produces a "sick" colony phenotype. The "healthy" phenotype is restored by introduction/transformation of a plasmid, integration vector or DNA fragment containing an intact *mdh* gene as well as a gene of interest. The intact *mdh* gene complements the non-functional *mdh* gene in the thermophilic host. This provides for a reliable and convenient means of detecting and confirming the integration of homologous, and associated heterologous, DNA into the chromosome of the thermophilic host. Maintenance of the intact *mdh* gene is required for healthy growth of the culture and, in turn, stabilizes the introduced gene of interest. In addition, by cloning the gene of interest adjacent to the *mdh* gene in the plasmid or integrative vector, any gene of interest can be introduced and maintained in a stable manner in *Thermus*. This is particularly useful for genes for which there is no convenient selection and this invention insures that a gene of interest will be stably maintained in the *Thermus* host, thereby overcoming a major problem in the production of biotechnology products.

As shown in Fig. 1, the *mdh* gene in a *Thermus* chromosome (wild type) is disrupted after transformation with pIGT-TMDH1 in a double cross-over event. pKII MDH, shown in Fig. 1, is a *Thermus* plasmid that carries a functional *mdh* gene downstream of a *Thermus* promoter. A multiple cloning site (MCS) site downstream

5 of an additional strong *Thermus* promoter allows for cloning and expression of genes of interest for the formation of biotechnology products. Any gene of interest can be cloned into the MCS and expressed by the strong *Thermus* promoter located immediately upstream. This vector and its derivatives are stably maintained in *Thermus* hosts that lack a functional *mdh* gene.

10 In accordance with one embodiment of this invention, the plasmid pKII MDH is modified to delete the *Thermus* replication genes. As a result, this plasmid can only replicate in *E. coli* but not in *Thermus* hosts. However, this plasmid can be introduced into *Thermus* hosts and because it contains regions of DNA that are homologous to regions of DNA in the chromosome of *Thermus*, homologous

15 recombination can result in the integration of this plasmid into the chromosome of *Thermus* allowing it to be replicated/maintained as a portion of the chromosome. This is a single crossover integration vector. A multiple cloning site downstream of a strong *Thermus* promoter allows for cloning and expression of genes for the formation of biotechnology products. Any region of homology of the *Thermus* chromosome can

20 be used provided it has a size equal to or greater than 50 bp of DNA. Similar integrative vectors can be constructed that contain two regions of homology with the *Thermus* chromosome flanking the *mdh* gene and the gene of interest. Such plasmids

can integrate into the chromosome of *Thermus* hosts by a double crossover event. Single crossover integrative plasmids result in the integration of the entire plasmid into the chromosome of the host while double crossover integrative vectors result in the integration of only those genes flanked by homologous sequences.

In this example, a *Thermus thermophilus* HB27 strain was constructed in which the malate dehydrogenase gene was deleted. Construction of this strain is shown in Fig. 2. To create the Δm*dh* strain of *T. thermophilus* HB27, we constructed an integration vector designated pUC-S KmA. A 2.3-kb PCR fragment containing a region spanning three separate genes, succinate coenzyme A ligase *(scsA)*, malate dehydrogenase *(mdh)*, and purine phosphoribosyltransferase *(gpt)*, was amplified from the *T. flavus* chromosome and cloned into pUC18. A 10-ul PCR amplification reaction was conducted in an Idaho Technologies Rapid Air Thermo-Cycler in the presence of 8% glycerol and 1% dimethyl sulfoxide. The PCR amplification program was run for 40 cycles at 94°C, 55°C, and 1 min of holding at 72°C. The 984-bp *mdh* gene is located near the center of this 2.3-kb fragment, so that the chromosomal regions that flank *mdh* are 780 bp (5') and 560 bp (3'). The entire coding sequence of the *mdh* gene was removed by restriction enzymes and replaced with a thermotolerant kanamycin resistance cassette (Km^r). This vector, designated pUC-SKmA, can replicate in *E. coli* but not in *Thermus*. However, homologous recombination between the *scsA* and *gpt* gene sequences allowed pUC-SKmA to integrate into the chromosome when it was used to transform *T. thermophilus* HB27. Transformants were screened at 55°C on TT rich medium supplemented with kanamycin (40 μg/ml).

Approximately 10^4 kanamycin-resistant transformants per μg of DNA were observed. Two distinct colony types arose after 5 days of incubation. The majority of the colonies were very small (0.1 to 0.5 mm) even after 5 days. A few colonies (60 to 100 CFU) were much larger (1.5 to 2.8 mm), the same size as wild-type *T. thermophilus* HB27 colonies. The two colony types were subcultured, and total DNA (plasmid and chromosomal) was dot blotted onto a nylon membrane.

The dot blot was probed with a digoxigenin-11-dUTP (DIG)-labeled *T. flavus mdh* gene. DNA prepared from the smaller colonies did not hybridize to the *T. flavus mdh* gene, whereas DNA harvested from the larger colonies hybridized to the *mdh* probe. The membrane was also probed with a DIG-labeled Km^r cassette, and both small and large colony types hybridized to it. Because malate dehydrogenase is a key enzyme in the tricarboxylic acid cycle, the colonies resulting from double-crossover integration events (Δmdh) are recognized by this small-colony phenotype. The larger Km^r colonies were single-crossover integration events in which the chromosomal *mdh* is intact and the entire plasmid is in the chromosome. The *T. thermophilus* Δmdh Km^r mutant strain was designated MM8-5. *T. thermophilus* Δmdh Km^r MM8-5 was used as a recipient in further transformation experiments.

In this example, *Thermus* vectors containing *mdh* as a reporter gene were constructed in accordance with one embodiment of this invention. The malate dehydrogenase *(mdh)* gene from *T. flavus* was amplified by PCR and cloned into *Thermus-E. coli* expression vector pTEXI. The expression vector pTEXI is capable of replication in both *Thermus* sp. and *E. coli*, and the promoter (J17) employed in

this expression vector functions in both bacterial hosts. J17 is a constitutive promoter

isolated from *T. thermophilus* chromosomal DNA. The expression vector containing

the *mdh* gene, designated pTEXI-mdh, is diagramed in Fig. 3A.

TABLE 1. Malate dehydrogenase activity of *Thermus* vector constructs

Strain	Promoter expressing *mdh*	MDH activity (U/mg)	
		25°C	50°C
HB27	Wild type	2.5 ± 0.12	19.9 ± 0.25
MM8-5/pSJ17mdhA*	J17	0.6 ± 0.01	5.5 ± 0.17
HB27/pTEX1-mdh	J17	3.7 ± 0.06	29.6 ± 1.39
MM8-5	None	0.0	0.5 ± 0.01
MM8-5/pTEX1-mdh	J17	4.2 ± 0.13	32.6 ± 0.72
MM8-5/pTEX1-D50-3	D50-3	0.3	2.7 ± 0.07
MM8-5/pTEX1-P2-300	P2-300	3.0 ± 0.07	23.2 ± 0.99

Activity values recorded are averages of three replicate samples from three separate experiments, for a total of
nine data points. Standard deviation is less than 5%. One unit of activity is defined as the amount of enzyme
needed to convert 1 μmol of NADH to NAD in 1 min. *, integrative vector

The J17 promoter from pTEXI-mdh was replaced by two constitutive *Thermus*

promoters isolated in our laboratory (D50-3 and P2-100). These promoters have low

and medium levels of expression, respectively, in *Thermus* sp. relative to J17. The

resulting plasmids were designated pTEX1-D50-3 and pTEX1-P2-100. An

integrative vector was constructed to examine the expression of the *mdh* gene under

control of the J17 promoter present as a single integrated copy. This construct was

designated pSJ17mdhA and is shown in Fig. 3B. pSJ17mdhA contains pUC19

sequences and can replicate in *E. coli*. pSJ17mdhA does not replicate in *Thermus* sp.

as a plasmid but integrates into the chromosome by a double-crossover event.

pSJ17mdhA has the J17 promoter-mdh gene cassette flanked by the *scsA* and *gpt*

chromosomal regions. A transcription termination sequence from the *T. flavus* phenylalanyl tRNA synthetase operon was cloned upstream of the cassette to prevent transcription read through from the native succinateCoA ligase/malate dehydrogenase operon promoter.

Plasmids pTEXI-mdh, pTEXI-D50-3, and pTEXI-P2-100 and the integrative vector pSJl7mdhA were transformed into MM8-5. Transformants were easily detected by the restoration of cultures to the wild-type or larger and faster-growing colonies by the expression of the malate dehydrogenase gene located on these expression vectors. Typically, *T. thermophilus* strain MM8-5 takes 4 to 5 days to form small visible colonies at 55°C in TT supplemented with 40 μg of kanamycin per ml. However, *T. thermophilus* MM8-5 transformants that received an expression vector carrying the *mdh* gene yielded larger colonies in 2 to 3 days.

Expression vector pTEXI-mdh and the alternative promoter pTEX derivatives are very stable in *T. thermophilus* MM8-5. After more than 20 generations of growth under nonselective conditions, pTEX plasmids were detected in all of the colonies examined (100 for each species). This result is expected because *T. thermophilus* MM8-5 cells that possess expression vectors containing the *mdh* gene grow more rapidly than plasmid-free cells that lack a functional *mdh* gene.

The levels of malate dehydrogenase being produced by plasmid and integrative expression vectors were evaluated in both *T. thermophilus* HB27 and MM8-5. Crude lysates prepared from each culture were assayed for enzyme activity at two temperatures (25°C and 50°C), and the results are shown in Table 1. *T.*

thermophilus MM8-5 had slight to no malate dehydrogenase activity, confirming complete deletion of the *mdh* gene from the chromosome of this strain. The activity observed in assays performed at 50°C reflect a slight amount of background due to the conversion of NADH to NAD by unidentified components of cell lysates rather than the malate dehydrogenase-dependent conversion of oxaloacetate and NADH to malate and NAD.

The malate dehydrogenase activity of crude extracts assayed at 50°C is on average nine times higher than the activity levels measured at 25°C. The data in Table 1 clearly indicate that promoters D50-3, P2-300, and J17 have different strengths, resulting in enzyme levels in MM8-5 strains that are 0.28, 1.16, and 1.65 times the level in wild-type *T thermophilus* HB27, respectively. Since each promoter is evaluated in identical genetic constructs that differ only by the promoter driving expression of the *mdh* gene, these levels serve to accurately quantify the strength of these promoters. Other strains whose enzyme activity is listed in Table 1 all use the same promoter, J17, to express the *mdh* gene in various backgrounds.

MM8-5/pS-J17mdh-A contains a single copy of the *mdh* gene integrated into the chromosome under the control of the J17 promoter. MM8-5/pTEXI-mdh contains the *mdh* gene under the control of the J17 promoter on a plasmid vector, and HB27/pTEXI-mdh contains two separate sources of the *mdh* gene, a wild-type *mdh* gene on the chromosome and the *mdh* gene under the control of the J17 promoter on a plasmid vector. Since MM8-5/pTEXI-mdh yields 32.6 U/mg of malate dehydrogenase per mg of protein at 50°C, it is unexpected that HB27/pTEXI-mdh,

which contains two separate copies of the *mdh* gene, a wild-type *mdh* gene on the chromosome as well as the *mdh* gene on a plasmid vector, shows nearly the same activity (29.6 U/mg).

5 In this example, integration vectors were constructed that allow for "visual detection" of DNA fragments inserted into *Thermus* sp. chromosomes. Successful transformants are recognized by the loss of pigment due to insertional inactivation of the pigment gene. This provides for a reliable and convenient means of detecting and confirming the integration of homologous DNA into the chromosome of thermophilic hosts.

10 A *Thermus thermophilus* HB27 strain was constructed in which the phytoene dehydrogenase (*phyD*) gene was deleted. The Δ*phyD* colonies are recognized by a white colony phenotype. Wildtype phenotype is restored by transformation with *Thermus* plasmids containing an intact *phyD* gene. The wildtype phenotype provides a screening tool for the introduction of plasmid DNA into 15 *Thermus sp.* and because *phyD* levels can be readily quantified, this host/vector system is a convenient tool for monitoring gene expression.

To create the Δ*phyD* strain of *T. thermophilus* HB27, we constructed an integration vector designated pGEM-phyD trunc. The plasmid is detailed in Figure 4. A PCR fragment containing the *phyD* gene was amplified from the *Thermus* 20 *thermophilus* HB27 chromosome and cloned into pGEM T-easy. The pGEM-phyD construct was restriction enzyme digested with two unique restriction enzymes, BglII and KpnI. The restriction termini overhands were blunted using a Klenow fragment

of DNA polymerase I. The blunt ends were ligated to each other. The *phyD* gene is inactive. In the construction, the 3'-region of *phyD* and the 5'-region of *phyD* are left intact. Homologous recombination between *phyD* sequences allowed integration into the chromosome when it was used to transform *T. thermophilus* HB27.

5   Transformants were screened at 55°C on TT rich medium. Approximately 1 x 10^2 white colony transformants per μg DNA were observed.

In this example, *Thermus* vectors containing *phyD* as a reporter gene were constructed. The phytoene dehydrogenase *(phyD)* gene from *Thermus thermophilus* HB27 was amplified by PCR and cloned into a *Thermus* and *E. coli*

10   expression vector pTEXI. The expression vector pTEXI is capable of replication in both *Thermus* sp. and *E. coli*, and the promoter (J17) employed in this expression vector functions in both bacterial hosts. J17 is a constitutive promoter isolated from *T. thermophilus* chromosomal DNA. This expression vector containing the *phyD* gene, designated pTEXI-phyD, is diagramed in Figure 5. A transcriptional

15   termination sequence from the *T. flavus* phenylalanyl tRNA synthetase operon was cloned upstream of the cassette to prevent transcription read through from the native succinate--CoA ligase/phytoene dehydrogenase operon promoter.

A *T. thermophilus* Δ*phyD* strain was used where the native *phyD* had been interrupted using a truncated and inactive *phyD* gene as a host for *Thermus*

20   plasmids expressing an intact *phyD* gene(pTEX1-phyD). The *phyD* gene can be used as a reporter gene to quantify promoter strength in *T. thermophilus*.

In this example, a gene expression system (pTEX) for *Thermus*

thermophilus HB27 was developed by using the β-galactosidase (*β-gal*) gene from

Thermus A4 sp. as the reporter gene. A PPKU, *T. thermophilus* HB27 strain was

constructed in which the *β-gal* gene was deleted in the chromosome using a double

crossover integration vector, as shown in Fig. 6. To test the reporter system, two

5   inducible promoters of *PdnaK* (coding for *dnaK* heat shock inducible protein induced

by shifting the incubation temperature from 70 to 85°C) and *Parg* (coding for

unknown protein in arginine operon induced with the addition of arginine to the

growth medium) and one carbon regulated promoter of key TCA cycle metabolic

pathway genes, *Pscs-mdh* (coding for succinyl-coA and malate dehydrogenase) were

10   cloned upstream of the reporter gene in pTEX in order to construct vectors pTEX7,

pTEX8, and pTEX9 respectively, as shown in Fig. 7. These plasmids were

transformed into *T. thermophilus* PPKU, a *Thermus thermophilus* HB27 *Δβ-gal* strain.

Blue-green phenotype with X-gal containing agar is shown by transformation with

Thermus plasmid vectors containing an intact *Thermus* A4 *β-gal* gene. Blue-green

15   phenotype in a strain provides a visual screening tool for the introduction of plasmid

DNA into *Thermus* sp. and the level of *β-gal* activity can be easily quantified,

facilitating gene expression studies in *Thermus*.

The bacterial strains and plasmids used in this study are listed in Table

2.

Table 2

Strain or Plasmid	Description
Bacterial strains	
E. coli DH5α *T. thyermophilus* HB27	Wild type
Plasmids	
pMK18 pUC18 pBGB3 pTEX1 pTEX4 pTEX7 pTEX8 pTEX9	*Thermus* cloning vector *Pslp* promoter with *Km* gene *Thermus* A4 *sp.* *β-gal* *P17* promoter with *Km* gene NdeI cloning vector *Pslp* promoter with *Km* gene *PdnaK* promoter with *β-gal* gene *pArg* promoter with *β-gal* gene *Pscs-mdh* promoter with *β-gal* gene

Thermus thermophilus HB27 was grown in TT rich medium (Lasa et al., "Development of *Thermus-Eschericia* Shuttle Vectors and Their Use for Expression of *Clostridium Thermocellum celA* Gene in *Thermus Thermophilus*" Journal of Bacteriology, 174:6424-6431,1992) and defined medium 162 (Degryse et al., "A Comparative Analysis of Extreme Thermophilic Bacteria Belonging to the Genus *Thermus*," Archives of Microbiology, 117:189-196, 1978). Arginine and uracil-free liquid medium with 20 mM pyruvate as the carbon source and 10 mM ammonium sulfate as the nitrogen source were employed for arginine induction experiments. Growth of *T. thermophilus* HB27 on single carbon sources was tested with minimal medium described in Fridjonsson et al., "The Structure of the Alpha-Galactosidase Gene Loci in *Thermus brockianus* IT1360 and *Thermus thermophilus* TH125", Extremophiles, 4:23-33, (2000). *Escherichia coli* DH5α was grown using nutrient broth or nutrient agar (Difco). Unless otherwise noted, kananycin was

included in liquid or agar medium at 40 µg ml^{-1} in experiments involving either *T. thermophilus* or *E.coli* cultures that were incubated at 55° and 37 °C, respectively.

β-galactosidase specific activities are determined as follows. Cell-free extracts are prepared for the β-galactosidase activity tests by growing *T. thermophilus*

5   HB27 cultures in TT media, at 55°C overnight, until mid-to late-log phase growth is achieved. When heat-shock is employed, cell cultures are incubated for 10 min at 85°C. When arginine is added, cell cultures are induced during a 4-hour period. Cell pellets are harvested by centrifugation at 10,000 x g for 10 minutes at room temperature, washed once with 0.1 M potassium phosphate (pH7.0) buffer and

10   resuspended in same buffer. The cell suspensions are disrupted by sonication using a Branson 350 sonifier (Branson Sonifer Co., Danbury, CT, USA) equipped with a microtip. The cell-free extracts are centrifuged at 10,000 x g for 15 minutes at room temperature to remove cellular debris. β-galactosidase activity is determined using spectrophotometric assay according to the manufacturer's protocol (Promega). One

15   unit of β-galactosidase activity is defined as 1n mol o-nitrophenol liberated from o-nitrophenol-β-D-galactoside min^{-1} at 37°C. Specific activity is expressed as β-galactosidase units (mg protein)$^{-1}$. Protein content is determined according to Smith, P. K.. Et al., "Measurement of Protein Using Bicinchoninic Acid", Analytical Biochemistry. 150, 76-85 (1985). The BCA (bicinchoninic acid) protein assay reagent supplied with

20   the system (Pierce Chemical Co., IL, USA) is used. Bovine serum albumin is used as a standard. β-galactosidase values are the averages of at least four experiments, with a variation of no more than 10% from the mean.

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Recombinant techniques are standard unless otherwise noted (Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbour, NY, 1989). Transformation of *Thermus* was carried out according to Koyama et al. "Genetic Transformation of the Extreme Thermophile *Thermus thermophilus* and of Other *Thermus* spp." Journal of Bacteriology 166:338-340, 1986. PCR amplification of the *β-gal* gene was performed with 1U of *Taq* DNA polymerase (Eppendorf), 20ng of plasmid pBGB3, 0.25 μM concentration of each primer, a 25μM concentration of dNTP mix (Life Technologies), and 1.5mM $MgCl_2$ in the buffer recommended by the manufacture. Amplification was achieved with 25 cycles of 30 sec of denaturation at 94°C, 30 sec of annealing at 55°C, and 2 min of extension at 72°C, plus an additional extention at 72°C for 7min with a Perkin Elmer thermal cycler.

T. thermophilus HB27 containing pTEX7, pTEX8 or pTEX9 that have the *β-gal* gene under the control of promoters *PdnaK, Parg,* and *Pscs-mdh,* respectively, were used to study gene expression. The effect of temperature shock, arginine concentrations, and various carbon sources was determined using *T. thermophilus* HB27 containing pTEX7, pTEX8 or pTEX9 , respectively. Detection of β-galactosidase activity performed with recombinant strains demonstrated that the reporter gene produced a functional β-galactosidase in *T. thermophilus* HB27. ß-gal activity in the arginine promoter construct increased from 6.5 U/mg to 9.75 U/mg after arginine addition and the *dnaK* operon promoter construct increased from 5.2 U/mg to 10.4 U/mg after heat shock induction. The different level of β-galactosidase activity has been demonstrated in the growth of diverse carbon sources with *scs-mdh*

promoter construct (pTEX9). These results validate the usefulness of these vectors for gene expression studies in *Thermus* and have contributed toward the development of a valuable thermophilic host/vector expression system.

In this example, a *T. thermophilus* HB27 host/vector expression system

5   is constructed. A series of promoters were cloned upstream of *Thermus* A4 β-galactosidase gene in order to test the newly developed reporter system. Fig. 7 summarizes the construction of the reporter system and the three test vectors developed in this study. Each promoter region was amplified by a PCR reaction (Table 3). Amplified promoters were cloned into pTEX4. A 1983-bp *β-gal* gene from

10   *Thermus* A4 *spp.* was amplified from pBGB3 vector using the following PCR primers: forward *β-gal* 5'-ACACACCATATGCTCGGCGTTTGCTATTACC-3' (S E Q   I D   N O :   1)   a n d   r e v e r s e   *β - g a l*   5 ' - GCGGCAGACCCGCCGCCGTACAAGCTTACACAC-3' (SEQ ID NO: 2). Restriction sites were added into the PCR primers to give 5'*Nde*I and 3'*Hind*III sites.

15   The *β-gal* gene PCR product was cloned into pTEX7, pTEX8, and pTEX9 vectors, respectively. Km^R gene cassette from pTEX1 was amplified by PCR using the f o l l o w i n g   P C R   p r i m e r s :   f o r w a r d   K m ^{R} 5 ' - ACACACTTTAAACATGGCCTAATGCCACCC-3' (SEQ ID NO: 3) and reverse Km^R 5'- TTGTGTTTTAAATCAAAATGGTATGCGTTT-3' (SEQ ID NO: 4) and

20   cloned into pTEX7, pTEX8, and pTEX9, respectively (Fig. 7).

Table 3. Primers for Amplification of Promoter Regions.

Promoters	Primers	
PdnaK	Forward	ACACACCTTAAGGGGTGTCCCCGGCGCGC
	Reverse	ACACACCATATGACGTCTTCACCTCGCC
Parg	Forward	ACACACCTTAAQGCTTAAGGAATTCTGGCCGCC
	Reverse	ACACACCATATGCCTTATCACCTTCCTTTT
Pscs-mdh	Forward	ACACACCTTAAGAATTGCTGGATGGCCTC
	Reverse	ACACACCATATGGACCCACCTCCCTTGCCG

Restriction sites were added into the PCR primers to give 5'*Afl*II and 3'*Nde*I sites.

In this example, a β-galactosidase mutant is constructed by double crossover for use as a recipient strain. To use *T. thermophilus* HB27 for expression of heterologous and homologous β-galactosidase, the *β-gal* gene was inactivated. An integration cassette was constructed in which the β-galactosidase gene was insertionally inactivated in vitro with a gene (*kan*) encoding a thermostable kanamycin resistance protein. By the construction, the 3'-region of *β-gal* and the 5'-region of *β-gal* were left intact to allow sequences for homologous recombination, but the middle of the gene was deleted. The *T. thermophilus* HB27 was transformed with a suicide integration vector and transformants were selected on kanamycin plates incubated at 55°C. Because suicide vectors cannot replicate in *Thermus*, resistant colonies should be those in which the integration cassette has inserted into the chromosome by homologous recombination between the *β-gal* flanking sequences in the plasmid and the chromosome. To verify the integration of the *kan* gene into the

β-galactosidase gene locus, chromosomal DNA from a transformant strain and the wild type were extracted and analyzed by PCR. Expected fragments were amplified according the length in Fig 6. The molecular analysis revealed the insertion of *kan* into the *β-gal* locus by homologous recombination, via double crossover, creating the

5   mutant strain PPKU.

Since the presence of glucose notably influenced the level of *mdh* gene expression in *E. coli*, we examined how carbon compounds affect the *Pscs-mdh* promoter-*β-gal* gene expression. *β-gal* expression under the control of the *Pscs-mdh* promoter varied over a 2.5-fold range (Fig. 8). Expression was lowest when a glucose

10   medium was used and highest when malate was used as the carbon source. Expression of the *mdh* gene is under catabolite control like *E. coli* by carbon type.

T. thermophilus HB27/pTEX8 was grown in arginine and uracil-free liquid medium to an OD_{600} of 0.6, and then various concentrations of arginine were added and the cultures were incubated 120 min at 65°C. The β-galactosidase

15   activities measured on crude extracts obtained from these cultures are shown in Fig. 9. An optimal concentration of arginine which induced expression of the *parg:: β-gal* fusion was 10 mM.

An object of this invention is to develop an efficient gene expression reporter system for *T. thermophilus* HB27. We examined the *β-gal* gene from

20   *Thermus* A4 sp. to determine whether it could be used as a reporter gene. Detection of β-galactosidase specific activity after this reporter gene was introduced on a plasmid indicated that the β-galactosidase produced by the new reporter system was

functional. This was confirmed by the 10-100 fold increase in β-galactosidase specific activities in strains containing the *Pdnak, Parg,* or *Pscs-mdh* promoters upstream of the reporter gene. The substantial differences in specific activity between the reporter systems with and without *Thermus* promoters also suggest that the sensitivity of the reporter system is adequate to monitor even weak promoters.

While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for the purpose of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein can be varied considerably without departing from the basic principles of this invention.